incubation mixture at zero incubation time. The fluorescence was measured with a Perkin-Elmer 650-10S instrument with excitation at 390 nm, emission at 440 nm, and excitation and emission slit widths of 2 nm. The instrument was standardized with authentic 7-hydroxycoumarin (Aldrich Chemical Co.) in 1.6 M NaOH–glycine buffer, pH 10.3.

Inhibitors were added in 20-μL aliquots from concentrated stock solutions in methanol. At least four assays at each substrate concentration in the presence and in the absence of inhibitors were performed.

Novel Immunosuppressive Agents. Potent Immunological Activity of Some Benzothiopyrano[4,3-c]pyrazol-3-ones

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A unique class of immunosuppressant agents has been found by the use of a mouse model of immunity. A number of benzothiopyrano[4,3-c]pyrazol-3-ones are potent inhibitors of both the humoral and cellular immune response developed in mice in response to El$_4$ tumor cells. These compounds exhibit a selectivity for inhibiting the humoral response. Structure–activity relationships and Hansch analyses of data from the mouse model are discussed. One of the most potent compounds, 2-(4'-chlorophenyl)benzothiopyrano[4,3-c]pyrazol-3-one (1, CP-17193), exhibits ED$_{50}$ values for inhibiting the humoral and cellular responses in the range of 0.5–2 mg/kg, po, in the mouse model and also inhibits antibody production to several other antigens and prolongs skin graft survival in mice. These compounds are shown in several ways to be distinct from the lymphocytotoxic drugs cyclophosphamide and azathioprine.

The pathological involvement of the immune response has been implicated in a number of diseases, such as rheumatoid arthritis, systemic lupus erythematosis, and certain forms of nephritis, arteritis, and anemia.[1,2] Therapy for such diseases could logically be directed toward controlling the aberrant immune response. However, most of the clinical agents presently known to affect immune function[3] are lymphocytotoxic due to their effects on the DNA of rapidly dividing cells. These drugs, for example, alkylating agents such as cyclophosphamide and antimetabolites such as azathioprine, were originally developed as agents to inhibit proliferation of malignant cells. Only secondarily were they found to inhibit the immune response. In view of their mechanisms of action and their effects not just on lymphocytes but on many other proliferating cells, it is not surprising that such agents have a limited therapeutic ratio and a number of toxic effects[4,5] on rapidly proliferating tissues such as the gastrointestinal mucosa, bone marrow, and hair follicles. The frequency and severity of clinical side effects has prevented widespread acceptance of these immunosuppressive agents[6] in spite of the fact that significant therapeutic effects have been noted in several disease states.[7-9]

In the present work, compounds with the obvious potential for acting as alkylating agents or as antimetabolites were deliberately avoided. Instead, novel heterocyclic structures capable of regulating the immune response were sought. In the absence of structural prototypes, this study began by examining a large number of structurally novel organic compounds in an assay of cellular and humoral immunity carried out in the mouse.[10] This screening technique has uncovered a number of benzothiopyrano-[4,3-c]pyrazol-3-ones which potently inhibit the immune response and form the basis for this report.

Chemistry. Although some benzothiopyrano[4,3-c]-pyrazoles are known,[11] no 3-oxo derivatives of this ring system appear to be known. Almost all of the benzothiopyrano[4,3-c]pyrazol-3-ones (compounds 1–21) shown in Table I were prepared by essentially one synthetic technique. Thus, combination of a hydrazine with 3-carbomethoxy-4-thiochromanone in the presence of acetic acid at elevated temperature gave fair yields of the title compounds (I).
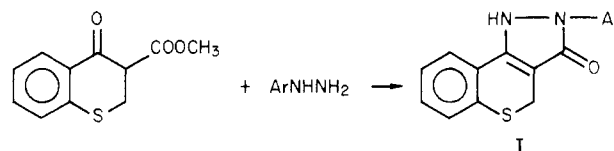

The Experimental Section provides details for the preparation of a representative compound, 1. Variations in reaction time or in equivalents of a particular hydrazine were made as appropriate to complete the reaction; Table I lists these specific conditions.

Data supporting the structural assignment for one representative compound (1, CP-17193) are detailed under Experimental Section. All analogues were analyzed (C, H, and N) and were compared to 1 by infrared and mass spectral data. Several compounds, titrated with NaOH in 2:1 dioxane–H$_2$O solvent, were shown to be of intermediate acid strength with pK_a values in the range of 7.1–7.7 (Table I). Thus, there appears to be only small effects on acidity resulting from changing the aryl substituent (Ar) in I from 4-methoxyphenyl (compound 8) to

(1) C. G. Cochrane and D. Koffler, *Adv. Immunol.*, 16, 185 (1973).
(2) N. J. Zvaifler, *Adv. Immunol.*, 16, 265 (1973).
(3) G. H. Hitchings and G. B. Elion, *Pharmacol. Rev.*, 15, 365 (1963).
(4) M. C. Berenbaum and I. N. Brown, *Immunology*, 7, 65 (1964).
(5) J. F. Bach, "The Mode of Action of Immunosuppressive Agents", Elsevier, New York, 1975.
(6) S. R. Kaplan and P. Calabresi, *N. Engl. J. Med.*, 289, 952 and 1234 (1973).
(7) Cooperating Clinics Committee of the American Rheumatism Association, *N. Engl. J. Med.*, 283, 883 (1970).
(8) A. D. Steinberg, H. B. Kaltreider, P. J. Staples, E. J. Goetzl, N. Talal, and J. L. Decker, *Ann. Intern. Med.*, 75, 165 (1971).
(9) S. N. Novak and C. M. Pearson, *N. Engl. J. Med.*, 284, 938 (1971).

(10) I. G. Otterness and Y.-H. Chang, *J. Clin. Immunol.*, 26, 346 (1976).
(11) For a review of benzothiopyrans, including certain benzothiopyrano[4,3-c]pyrazoles, see S. W. Schneller, *Adv. Heterocycl. Chem.*, 18, 88 (1975).
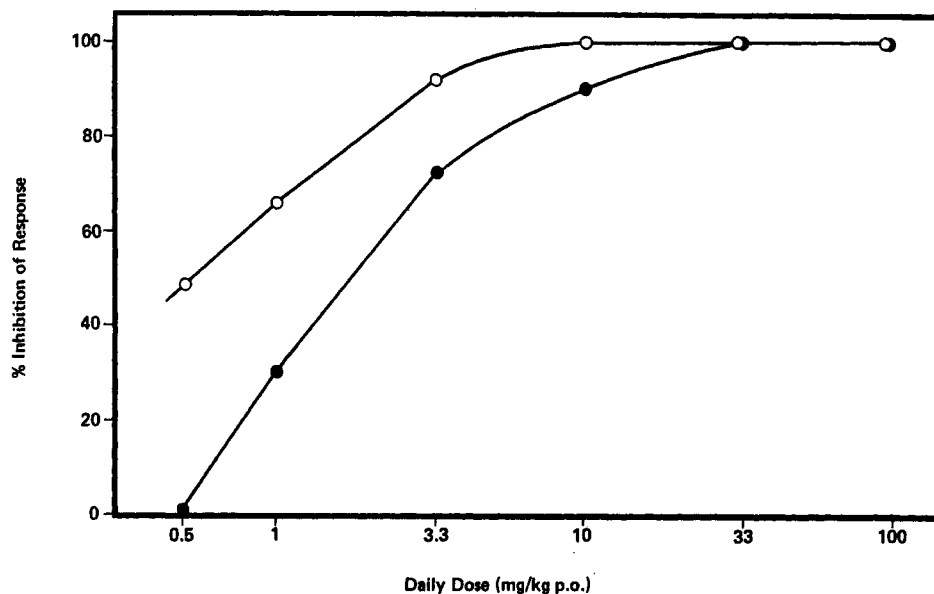
Figure 1. The immunosuppressive activity of compound 1 on the response of Balb/c mice to El$_4$ tumor cells. The percent inhibition of the cellular immune response (closed circles) and the humoral immune response (open circles) is shown as a function of the daily dose.

phenyl (compound 2) to dichlorophenyl (compound 10).

The normally pale yellow 1,2,3,4-tetrahydro compounds (1–21) showed a tendency to oxidize slowly to orange-red 2,3-dihydro compounds, such as 22. In fact, facile conversion of 1 to 22 was achieved by an *o*-chloranil oxidation
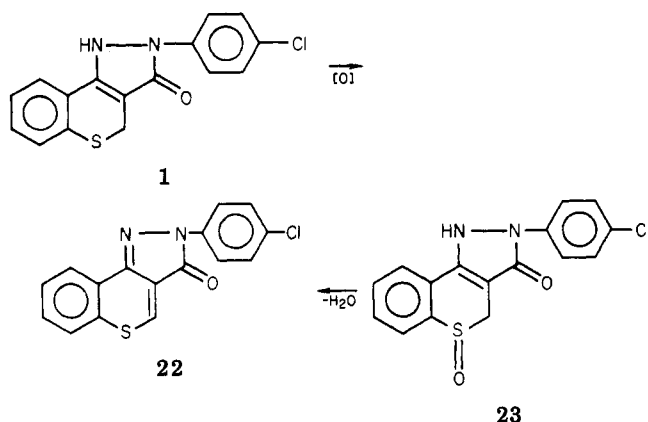
of 1 or by allowing 1 to stand in Me$_2$SO solution for several days. In addition, dehydration of sulfoxide 23 to 22 was achieved in warm acid solution.

Discussion

Several of these compounds are quite potent immunosuppressant agents, the most active (1) being effective at a dose of less than 1 mg/kg, po. This activity was not accompanied by lethality, since the LD$_{50}$ in mice was found to be >400 mg/kg, po, and compound 1 was tolerated on chronic administration to the same species for 14 days with no bone marrow depression, no loss of cellularity in the spleen or thymus, and no abnormal pathology at doses up to 400 mg/kg, po. These data should be contrasted with our results[10] for cyclophosphamide and azathioprine in BALB/c mice where the ED$_{50}$ for inhibition of the cellular immune response to El$_4$ cells (8 and 30 mg/kg, po, respectively) and the chronic LD$_{50}$ (unpublished) obtained after 14 days dosing (~90 and ~125 mg/kg, po, respectively) differ by a factor of 10 and 4, respectively. For 1 the ED$_{50}$ and LD$_{50}$ differ by a factor of greater than 400. With both cyclophosphamide and azathioprine, depressive effects on spleen cellularity are readily noted at therapeutic doses.

In addition, cyclophosphamide[12] and azathioprine[13] are both known mutagenic agents. In a system similar to that used to demonstrate the mutagenicity of cyclophosphamide,[12] e.g., the number of revertants to histidine independence for several *Salmonella typhimurium* strains, compound 1 was found to lack mutagenic potential.[14] Also, in contrast to azathioprine and cyclophosphamide,[15] doses of 1 up to 300 mg/kg, po, did not cause chromosomal breakage in mouse bone marrow.[14]

The sulfoxide 23, prepared by oxidation of 1 and considered to be a possible in vivo metabolite of 1, is also a potent immunosuppressant. Sulfoxide 23 was rapidly dehydrated to the highly active 22, a compound which also forms readily from 1 under mild oxidative conditions. The possibility that 22 may be an active metabolite of 1 in vivo must be considered.

A group of compounds from this initial series was selected for more extensive pharmacologic testing in order to examine in greater detail the structural features contributing to activity. These compounds were chosen to span the range from very weak to very potent immune activity as evidenced by the initial testing at a dose of 10 mg/kg. Activity was measured for each compound so that generally three points could be used to determine a dose–response line from which an ED$_{50}$ was calculated. Table II records the ED$_{50}$ values for inhibition of both the cellular and humoral immune response for these compounds.

The data of Table II demonstrate that these compounds generally possess a relative selectivity for inhibition of the humoral immune response. A comparison of ED$_{50}$ values for the series suggests that on the average it takes 2.6 ± 1.1 times more compound to achieve the cellular ED$_{50}$ than the humoral ED$_{50}$. To further illustrate this selectivity for the humoral response, a complete dose–response curve is
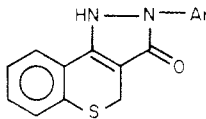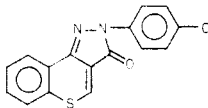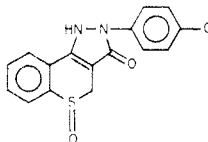
(12) J. McCann, V. Simmon, D. Streitwieser, and B. N. Ames, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3190 (1975).

(13) W. T. Speck and H. S. Rosenkranz, *Cancer Res.*, **36**, 108 (1976).

(14) H. E. Holden, Pfizer Central Research, unpublished results.

(15) (a) S. F. Tolchin, A. Winkelstein, G. P. Rodnan, S. F. Pan and H. R. Nankin, *Arthritis Rheum.*, **17**, 375 (1974); (b) P. K. Datta and E. Schleiermacher, *Mutat. Res.*, **8**, 623 (1969); (c) M. K. Jensen, *Int. J. Cancer*, **5**, 147 (1970).

Table I. Chemical Characteristics and Immunological Activity of Benzothiopyrano[4,3-c]pyrazol-3-ones

compd	Ar	yield, %	reaction time, h	reaction temp, °C	mp, °C	crystn solvent[a]	formula[b]	pK_a[c]	immunological act.:[i] % suppression of the response to El_4 cells — cellular response ± SE	humoral response
1	4-ClC_6H_4	70	1	180	235–237	I	$C_{16}H_{11}ClN_2OS$	7.56	>90	>90
2	C_6H_5	48	1	115	208–210	I	$C_{16}H_{12}N_2OS$	7.48	1 ± 2	j
3	4-BrC_6H_4	23	2	115	239–241	I	$C_{16}H_{11}BrN_2OS$	7.13	84 ± 2	>90
4	4-FC_6H_4	48	10	150	193–196	Et	$C_{16}H_{11}FN_2OS$		86 ± 1	>90
5	2-ClC_6H_4	34[d]	12	180	233–235	B	$C_{16}H_{11}ClN_2OS$	7.74	8 ± 1	45
6	3-ClC_6H_4	50	30	180	202–204	Et	$C_{16}H_{11}ClN_2OS$		16 ± 6	67
7	4-$CH_3C_6H_4$	44	2	115	239–241	Et	$C_{17}H_{14}N_2OS$	7.64	19 ± 3	46
8	4-$CH_3OC_6H_4$	35	2	130	175–179	E	$C_{17}H_{14}N_2O_2S$	7.76	−11 ± 3	−6
9	3,4-$Cl_2C_6H_3$	30	2	115	219–221	I	$C_{16}H_{10}Cl_2N_2OS$		62 ± 1	87
10	2,3-$Cl_2C_6H_3$	39[d]	12	180	228–230	Et	$C_{16}H_{10}Cl_2N_2OS$	7.28	5 ± 1	51
11	2,4-$Cl_2C_6H_3$	54	35	180	274–276	Et	$C_{16}H_{10}Cl_2N_2OS$		−4 ± 1	20
12	3,5-$Cl_2C_6H_3$	42	22	180	245–247	Et	$C_{16}H_{10}Cl_2N_2OS$		19 ± 1	73
13	2,6-$Cl_2C_6H_3$	22[d]	63	180	239–241	Et	$C_{16}H_{10}Cl_2N_2OS$		1 ± 3	4
14	4-$CF_3C_6H_4$[e]	14	4	180	282–284	I	$C_{17}H_{11}F_3N_2OS$		71 ± 2	>90
15	4-$NO_2C_6H_4$	10	6	130	246 dec	C–M	$C_{16}H_{11}N_3O_3S$		27 ± 1	j
16	4-$CH_3SO_2C_6H_4$	8	11	160	172–176	Et	$C_{17}H_{14}N_2O_3S_2$[f]		1 ± 2	28
17	H	42[d]	10	130	125–127	C–I	$C_{10}H_8N_2OS$		1 ± 1	j
18	CH_3	27[d]	7	115	198–200	I	$C_{11}H_{10}N_2OS$		14 ± 5	−3
19	CF_3CH_2	38	4	120	184–187	B	$C_{12}H_9F_3N_2OS$		−2 ± 4	17
20	$CH_2C_6H_5$	44[d]	25	160	174–176	I	$C_{17}H_{14}N_2OS$		−3 ± 5	0
21	2-pyridyl	26	11	160	126–130	I	$C_{15}H_{11}N_3OS$		2 ± 4	−19
22		84[g]	1.5		230–232	B or D	$C_{16}H_9ClN_2OS$		63 ± 4	>90
23		88[h]	3.5		193 dec		$C_{16}H_{11}ClN_2O_2S$		>90	>90
cyclophosphamide									52 ± 2	>90
6-mercaptopurine									63 ± 1	52
azathioprine									−3 ± 1	6

[a] I = *i*-PrOH; Et = thorough trituration with Et_2O; B = C_6H_6; E = EtOH; C = $CHCl_3$; M = MeOH; D = Me_2SO. [b] Except where noted, satisfactory analyses for C, H, and N were obtained for all compounds. [c] Titrated in 2:1 dioxane-H_2O using 0.50 N NaOH. [d] To complete these reactions, an additional 0.25–0.50 equiv of the hydrazine was required. [e] The requisite 4-$CF_3C_6H_4NHNH_2$·HCl was prepared in 14% yield by the method of A. Léspagnol, D. Bar, E. Debruyne, and D. Savage, *Bull. Soc. Chim. Fr.*, 490 (1960), and was used without further purification. [f] Calcd: C, 56.96; H, 3.94; N, 7.82. Found: C, 56.04; H, 4.78; N, 7.72. [g] Prepared by two routes; see Experimental Section. [h] See Experimental Section. [i] Compounds administered to mice at 10 mg/kg, po. [j] Data on the humoral response for this compound were considered unreliable in this determination.

shown (Figure 1) for the 4'-chloro derivative (1). At all doses below maximal inhibition, the degree of suppression of the humoral response exceeds that of the cellular response.

The ten compounds from Table II were subjected to a Hansch analysis.[16,17] Correlations were sought between immunological activity and several parameters using all ten compounds from Table II for the humoral data but omitting compound 16 from the cellular data analysis.

The parameters π, π^2, π_-, π_-^2, Hammett's σ value, molecular weight (MW), molar refractivity (MR), inductive effect (F), and a resonance parameter (R) were employed in a stepwise regression analysis program using published values for the above parameters from Norrington et al.[18] Results of these analyses are recorded in eq 1 and 2, to-

Humoral Activity

$$\log (1/ED_{50}) = 3.30 + 1.13\ (\pm 0.57)\ \pi_- +$$
$$2.02\ (\pm 1.88)\ F - 0.79\ (\pm 0.62)\ \pi_-^2 \quad (1)$$
$$n = 10;\ s = 0.51;\ r = 0.88$$

(16) Computer-assisted Hansch analyses were carried out by Dr. B. Dominy of the Technical Information Department of Pfizer Central Research. The authors thank Dr. Dominy for his helpful discussions of results.

(17) C. Hansch, A. Leo, S. H. Unger, K. H. Kim and D. Nikaitani, *J. Med. Chem.*, 16, 1207 (1973).

(18) F. E. Norrington, R. M. Hyde, S. G. Williams, and R. Wooton, *J. Med. Chem.*, 18, 604 (1975).

Cellular Activity

$$\log (1/ED_{50}) =$$
$$3.52 + 2.07 \; (\pm 1.58) \; \pi_- - 0.79 \; (\pm 0.76) \; \pi_-^2 \quad (2)$$

$$n = 9; \; s = 0.59; \; r = 0.76$$

gether with the number of data points (n) used in each analysis, the standard deviation (s), and the correlation coefficient (r).

These data, based on a limited number of analogues available for this analysis, indicate that π_- values produced better correlations, with eq 1 accounting for about 78% of the variability in the biological data. The humoral activity (eq 1) is correlated to an inductive effect (F), while the cellular activity is not. The parameters F and π_- were found to cross correlate to the extent of 0.6; however, if compound 16 is omitted from the analysis, the F term is no longer significant in eq 1. By omitting compound 16, eq 3, now not very different from eq 2, was obtained for the humoral response.

Humoral Activity

$$\log (1/ED_{50}) =$$
$$3.77 + 2.19 \; (\pm 0.74) \; \pi_- - 0.78 \; (\pm 0.71) \; \pi_-^2 \quad (3)$$

$$n = 6; \; s = 0.55; \; r = 0.82$$

Conclusion

The series of compounds reported here are unique among the known immunosuppressive drugs. Although these compounds are potent suppressors of the immune response to El_4 cells in mice, they differ from both cyclophosphamide and azathioprine in that they lack bone marrow depressive effects (in mice after 14 days at doses up to 400 mg/kg, po) and are free of mutagenic activity. Oxisuran,[19] another low-molecular-weight suppressant of cell-mediated immunity, appears to be free of lymphocytotoxic effects, but this drug has been reported to act indirectly via adrenal stimulation[20] and in our mouse El_4 system fails to suppress either the cellular or humoral immune response at oral doses up to 100 mg/kg. Our mouse El_4 system is not inhibited by an oral dose of 500 mg/kg of hydrocortisone or corticosterone and, therefore, is not expected to detect agents which act through stimulation of the adrenal cortex. In addition, compound 1 retains immunosuppressant activity when tested in the El_4 system employing adrenalectomized mice.

The potency of the 4′-Cl derivative (1) is greater than either cyclophosphamide or azathioprine in the El_4 mouse model of the immune response. Moreover, the immunosuppressive activity of 1 extends beyond the El_4 model, since we have found it to inhibit the immune response to sheep erythrocytes, albumin, polyvinylpyrrolidinone, and lipopolysaccharide from *Escherichia coli* in mice and to prolong skin graft survival in mice. When examined in standard animal models of inflammation, compound 1 proved to be inactive in the rat paw edema model[21] (100 mg/kg, po) but active in the rat adjuvant arthritis model[22] (10 mg/kg, po). Compound 1 showed no lethal effects when administered orally even at multiple high doses to

Table II. Immune Activity of Benzothiopyrano[4,3-c]pyrazol-3-ones

no.[a]	X	dose, mg/kg po, required to inhibit the immune response 50%[b]	
		cellular response	humoral response
1	4-Cl	2	0.5
9	3,4-Cl$_2$	3.0	1.0
3	4-Br	3.3	2.0
4	4-F	5.5	3.3
14	4-CF$_3$	6.5	6.0
12	3,5-Cl$_2$	31	8.0
6	3-Cl	45	9.0
2	H	130	70
8	4-OCH$_3$	240	150
16	SO$_2$CH$_3$	>300[c]	170

[a] The number corresponds to the associated number in Table I. [b] The immune activity was determined according to the method described in the text at several doses such that generally two doses were found that gave, respectively, greater than and less than 50% inhibition. The dose for 50% inhibition was determined by linear interpolation between these two doses. [c] Fifty percent inhibition of the cellular response was not obtained at the highest dose tested, 300 mg/kg, po.

Table III. Data for Regression Analyses

no.	inhibn of immune response; $\log (1/C)^a$					π_-	F
	humoral			cellular			
	exptl	calcd[b]	calcd[c]	exptl	calcd		
1	5.80	5.07	5.13	5.20	4.77	0.93	0.69
9	5.54	5.24	5.05	5.07	4.54	1.97	1.37
3	5.25	5.05	5.25	5.04	4.86	1.13	0.73
4	4.96	5.02	4.37	4.73	4.09	0.31	0.71
14	4.76	4.89	5.21	4.73	4.83	1.05	0.63
12	4.64	4.99	4.95	4.05	4.41	2.08	1.36
6	4.54	5.01	5.20	3.84	4.82	1.04	0.68
2	3.60	3.30	3.77	3.33	3.52	0	0
8	3.32	3.99	3.49	3.11	3.26	-0.12	0.41
16	3.32	3.15				-1.02	0.90

[a] The ED_{50} values from Table II have been converted to molar concentrations (C). [b] Calculated from eq 1. [c] Calculated from eq 3.

mice (400 mg/kg), rats (125 mg/kg), hamsters (33 mg/kg), and dogs (10 mg/kg). However, the compound was lethal to rabbits. No gross lesions were observed in rats and mice after multiple doses. Rats, but not mice, exhibited some vacuolization of hepatocytes and focal areas of liver necrosis.

Experimental Section

Melting points were determined in a Thomas-Hoover capillary melting point apparatus and are uncorrected. Thin-layer chromatography (TLC) was carried out on glass plates (EM Laboratories) coated with silica gel 60 F-254 and visualized under ultraviolet light (254 nm). Potentiometric titrations were carried out in 2:1 dioxane–H_2O (v/v) solvent using a Beckman Model G pH meter and standard 0.5 N NaOH. The apparent pK_a values correspond to the pH values at the half-neutralization point in these titrations. A Varian A-60 spectrometer (Me$_4$Si standard) was used to measure NMR spectra, and mass spectra were determined on a Hitachi Perkin-Elmer Model RMU-6E. IR spectra were determined in KBr pellets. Analyses were carried out by

(19) H. H. Freedman, A. E. Fox, J. Shavel, Jr., and G. C. Morrison, *Proc. Soc. Exp. Biol. Med.*, **139**, 909 (1972).

(20) H. van Dijk, I. A. Bakker, J. Testerink, N. Bloksma, and J. M. Willers, *J. Immunol.*, **115**, 1587 (1975).

(21) C. A. Winter, E. A. Risley, and G. W. Nuss, *Proc. Soc. Exp. Biol. Med.*, **111**, 544 (1962).

(22) D. T. Walz, M. J. DiMartino, and A. Misher, *Ann. Rheum. Dis.*, **30**, 303 (1971).

the Physical Measurements Laboratory of Pfizer Central Research. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements are within ±0.40% of the theoretical values.

All of the hydrazines required as starting materials were purchased from Aldrich Chemical Co., except as noted in Table I. Most hydrazines were purchased as HCl salts and were converted to the free hydrazines by partitioning the salts between aqueous KOH/ether, drying (Na_2SO_4) the ether extracts, and then removing the solvent under reduced pressure. The hydrazines were then used immediately.

The general method for preparing the title compounds is illustrated below for compound 1; where appropriate, variations in reaction time, temperature, or in molar equivalents of a particular hydrazine are indicated in Table I.

2-(p-Chlorophenyl)-1,2,3,4-tetrahydro[1]benzothio-pyrano[4,3-c]pyrazol-3-one (1).[23] A mixture of 60 g (0.270 mol) of 3-carbomethoxy-4-thiochromanone,[24] 42.4 g (0.297 mol) of p-chlorophenylhydrazine, and 18 mL of HOAc under a N_2 atmosphere was heated at 115 °C in an oil bath for 30 min. The yellow solid was further heated to 180 °C for 1.5 h and then cooled and placed under vacuum to distill off excess HOAc. The residual semisolid was triturated repeatedly with a large volume of ether, filtering the suspension each time. The crude product (71 g) was dissolved in 3 L of i-PrOH, concentrated to 1.5 L, and cooled to yield 54 g (64%) of 1: mp 235–237 °C; IR 3.65 (br, NH), 6.12, 6.26, 7.63, 9.23, 12.1 nm; mass spectrum, m/e 314 (calcd 314) with appropriate Cl isotope distribution; NMR (Me_2SO-d_6) τ 6.0 (s, 2 H, CH_2), 2.1–2.8 (m, 8 H, aromatic protons).

On prolonged standing, samples of 1, either in the solid state or in solution, were found (by TLC using either 85:15 benzene/HOAc or $CHCl_3$ as eluent) to produce small amounts of the oxidized compound 22.

2-(p-Chlorophenyl)-2,3-dihydro[1]benzothiopyrano[4,3-c]pyrazol-3-one (22). Two different procedures were employed for the preparation of compound 22.

Method A. Oxidation of 1. To a solution of 2.0 g (0.0063 mol) of compound 1 in 20 mL of Me_2SO was added 1.55 g (0.0063 mol) of o-chloranil (Aldrich Chemical Co.). An orange precipitate appeared immediately and became heavier upon stirring the reaction at room temperature. After 1.5 h, the solid was filtered, rinsed with cold benzene, and dried in vacuo to yield 1.68 g (84%) of 22: mp 229–231 °C; IR 5.95 nm (C=O); mass spectrum, m/e 312 (calcd 312) with appropriate Cl isotope distribution; NMR ($DCCl_3$ at 100 MHz on a Varian XL-100-15 spectrometer) τ 1.38 (s, 1 H, CH=), 1.5–1.3 (m, 1 H, H-6), 1.82 (d, 2 H, J = 4.5 Hz), 2.56 (d, 2 H, J = 4.5 Hz, 4-ClC_6H_4 protons), 2.5–2.3 (m, 3 H, H-7, H-8, H-9).

Method B. Dehydration of Sulfoxide 23. To 0.050 g (0.00015 mol) of compound 23 in 5 mL of HOAc was added 5 drops of 12 N HCl. The suspension rapidly cleared to a yellow solution and, after 3.5 h at 85 °C, was completely converted (TLC evidence, 85:15 benzene/HOAc as eluent) to a new, less polar, visible yellow spot on TLC. Upon cooling of the reaction, an orange solid precipitated. The solid was filtered and dried to yield 0.030 g (64%) of 22, mp 231–232 °C, identical by IR, mass spectrum, and combustion analysis with the material isolated from method A.

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(23) Initial synthesis of this compound (CP-17 193) was accomplished by Dr. James F. Muren of Pfizer Inc.
(24) T. Moriwake, *J. Med. Chem.*, **9**, 163 (1966).

2-(p-Chlorophenyl)-1,2,3,4-tetrahydro[1]benzothio-pyrano[4,3-c]pyrazol-3-one 5-Oxide (23). To a suspension of 0.50 g (0.0016 mol) of compound 1 in 8.3 mL of HOAc at 10–15 °C was added 3 mL of 30% H_2O_2. After 3.5 h, TLC (85:15 benzene/HOAc) indicated only a new, more polar material. The suspension was filtered and rinsed with HOAc, and the white solid was dried in vacuo over P_2O_5 to yield, in two crops, 0.46 g (88%) of 23: mp 193 °C dec; IR 6.15 (C=O), 10.3 nm (S=O); NMR (Me_2SO-d_6) τ 5.8 (s, 2 H, CH_2), 2.15–1.9 (m, 9 H, aromatic protons and NH). This product was found to be unstable in the presence of acid (see method B).

Pharmacology. Assessment of immunosuppressant activity in mice was based upon inhibition of the cellular and humoral immune response to El_4 tumor cells according to our previously published method.[10] Groups of 10 Balb/c mice were immunized on day 0 by intraperitoneal injection with 10^7 cells from the murine ascites tumor El_4. The tumor was maintained by passage in syngenic adult C57Bl/6 mice. Drug was administered orally dissolved in water with the addition of a small amount of sodium hydroxide to permit solution (circa pH 8.5). Drug-treated groups were dosed once daily from day 0 to day 9. One day 10, both control (saline treated) and drug-treated groups were assayed for the development of humoral and cellular immunity against El_4 cells.

Humoral immunity was measured using complement-dependent lysis of ^{51}Cr-labeled El_4 cells. Cells, 1×10^7, were labeled with 60 μCi of ^{51}Cr in 10% CO_2 for 30 min at 37 °C, and then the labeled cells were washed twice with 45 mL of cold Hank's minimum essential media. Lysis was carried out using 0.5 mL of a dilution of sera from a control or drug-treated group, 0.2 mL of 2.5×10^5 labeled El_4 cells, and 0.3 mL of a one-fortieth dilution of rabbit complement previously absorbed with El_4 cells. The dilution giving 50% of maximal lysis of El_4 cells was determined from a linearized plot of dilution vs. percent lysis and was taken as the titer of the sera as previously described.[10] Drug effects are computed as percent inhibition of the control titer.

The cellular immune response was measured by the specific lysis of El_4 cells caused by spleen cells from sensitized mice. Spleens were minced, ground in a mortar and pestle, filtered, washed twice in Hank's minimal essential medium, and resuspended to a concentration of 1.5×10^7 viable cells/0.5 mL. A mixture of 0.5 mL of spleen cells and 0.5 mL of labeled El_4 cells (0.5×10^5) was incubated in a 35×10 mm petri dish for 3 h at 37 °C in a 10% CO_2 atmosphere on a rocker platform at 8 reciprocations/min. The cells were collected and centrifuged, the radioactivity in the supernatant and pellet was counted in a gamma counter (Nuclear of Chicago Model 4230), and the percentage lysis was determined after correction for spontaneous release. Drug effects are recorded as percent inhibition of the control lysis.

Immune activity for this series of compounds was first examined by determining relative inhibition of the cellular and humoral immune response at a daily dose of 10 mg/kg, po. Results of this initial screening effort are recorded in Table I.